Video Article

A Rapid Automated Protocol for Muscle Fiber Population Analysis in Rat Muscle Cross Sections Using Myosin Heavy Chain Immunohistochemistry

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Abstract

Quantification of muscle fiber populations provides a deeper insight into the effects of disease, trauma, and various other influences on skeletal muscle composition. Various time-consuming methods have traditionally been used to study fiber populations in many fields of research. However, recently developed immunohistochemical methods based on myosin heavy chain protein expression provide a quick alternative to identify multiple fiber types in a single section. Here, we present a rapid, reliable and reproducible protocol for improved staining quality, allowing automatic acquisition of whole cross sections and automatic quantification of fiber populations with ImageJ. For this purpose, embedded skeletal muscles are cut in cross sections, stained using myosin heavy chains antibodies with secondary fluorescent antibodies and DAPI for cell nuclei staining. Whole cross sections are then scanned automatically using a slide scanner to obtain high-resolution composite pictures of the entire specimen. Fiber population analyses are subsequently performed to quantify slow, intermediate and fast fibers using an automated macro for ImageJ. We have previously shown that this method can identify fiber populations reliably to a degree of ±4%. In addition, this method reduces inter-user variability and time per analyses significantly using the open source platform ImageJ.

Video Link

The video component of this article can be found at https://www.jove.com/video/55441/

Introduction

Skeletal muscle composition undergoes profound changes during physiological processes such as aging[1,2], exercise[3,4,5,6,7], or pathophysiological processes such as disease[8,9,10] or trauma[11]. Hence, several fields of research concentrate on the structural effects of these processes to understand functional changes. One of the key aspects determining muscle function is the composition of muscle fibers. Muscle fibers express different myosin heavy chain (MHC) proteins and are thereby classified into slow, intermediate, or fast fibers[7,12,13,14,15,16,17]. Physiologically, muscles have different muscle fiber compositions depending on their function in the body. Using muscle fiber typing, fiber populations can be quantified to identify adaption to physiological or pathophysiological processes[7,17]. Historically, a number of time-consuming methods have been applied to differentiate between muscle fiber types. For this purpose, muscle fibers were classified either by reactivity of myosin ATPase at various pH levels or muscle enzyme activity. As different fiber qualities could not be assessed in a single section, multiple cross sections were required to identify all muscle fibers and allow manual quantification[14,16,17,18,19,20,21,22]. In contrast, recent publications used immunohistochemistry (IHC) against myosin heavy chain protein to rapidly stain multiple fiber types in a single cross sections. Based on the advantages of this procedure, it is now considered the gold standard in muscle fiber population analysis[19,23,24]. Using improved IHC staining protocols, we were recently able to show that the fully automatic acquisition of whole muscle cross sections and subsequent automatic muscle fiber quantification is feasible using the open source platform ImageJ. Compared to manual quantification, our procedure provided a significant decrease in time (approximately 10% of manual analyses) required per slide while being accurate to ±4%[25].

The overall goal of this method is to describe a rapid, reliable, user-independent guide to automatic muscle fiber quantification in whole rat muscles using an open source platform. In addition, we describe potential modifications that would permit its use for other specimens such as mice or human muscles.

Protocol

All procedures including animal subjects were conducted in compliance with the principles of laboratory animal care as recommended by FELASA[26]. Approval was obtained prior to the study by the institutional review board of the Medical University of Vienna and the Austrian Ministry for Research and Science (BMWF: Bundesministerium fuer Wissenschaft und Forschung, reference number: BMWF-66.009/0222-WF/II/3b/2014).

1. Muscle Harvest

NOTE: A previous publication by Meng *et al.*[27] is available describing the correct freezing of muscle specimens in great detail.

1. **Obtain entire muscles or sufficient tissue to acquire entire cross sections immediately after euthanasia of the animals.**
    1. Remove the entire muscle from an anesthetized or euthanized rat. Remove all connective tissue and tendons surrounding the muscle with forceps and scissors. For anesthesia or euthanasia, follow the international guidelines of FELASA[28].
2. Weigh muscle using a calibrated precision scale. This quick step allows comparative analyses between muscle samples, especially after interventional procedures.
3. Put muscle into a vessel, filled entirely with O.C.T. compound, according to the size of the muscle with a safety margin of approximately 1 mm to all sides (*e.g.*, made of aluminum foil).
4. Freeze it in approximately 2 min precooled isopentane using liquid nitrogen. The isopentane must begin to show white crystals at the bottom of the container.
   NOTE: This step is essential for preserving the correct architecture of the muscle and thereby allows the correct staining and fiber analyses[27].
5. Store samples conserved in O.C.T. for at least 24 h at -80 °C. Samples can, however, be stored at -80 °C for several months or even years, if correctly cooled.
6. Cut 10 µm transverse sections from the midportion of the muscle at -20 °C using a cryotome. If cutting at 10 µm is not feasible, increase the thickness in steps of 2 µm up to a maximum of 20 µm. Sufficiently sharp cutting blades are essential for this step.
7. Apply the sections to adhesive slides by approximating the slides to the cross sections. The cross sections will automatically stick to the adhesive slides. Store the slides at -20 °C overnight before staining.

2. Staining

NOTE: A large number of antibodies against myosin heavy chain proteins are available; however, high quality antibodies are essential for automated acquisition and analyses. For dilutions and reference to antibodies, see **Table 1**. For a spreadsheet file to calculate the correct dilutions and see the number of required solution quantities, see Supplemental File 1.

1. Thaw and air-dry the frozen muscle sections for 10 min before the staining procedure.
2. Wash slides carefully with phosphate buffered saline (PBS) + 0.05% Triton X for 10 min and then for 5 min. Triton has been shown to improve the staining quality significantly. See the discussion for further details.
3. Let sections air-dry for 2 min and outline individual cross sections on each slide using a hydrophobic pen (to minimize required amount of antibody) and allow additional drying for 15 min.
4. Block all slides with PBS + 0.05% Triton X (PBST) containing 10% goat serum for 1 h at room temperature.
5. Dilute all primary antibodies (**Table 1**) in a cocktail of PBS + 0.05% Triton X (PBST) containing 10% goat serum. Mix sufficiently before application. Triton X is essential for equal staining of the whole cross sections. Calculate approximately 30 µL of primary antibody cocktail per cross section.
6. For steps 2.7-2.13 ensure sufficient protection from light. Dimmed room lights and covered staining trays provide adequate protection. Additionally, fill the staining tray with fluid at the bottom to provide a humid environment during staining and prevent drying.
7. Apply primary antibody (**Table 1**) cocktails to the slides for 1 h in a humid staining tray on room temperature.
8. Wash slides with PBS + 0.05% Triton X for 10 min and then with new PBST for 5 min.
9. Dilute secondary antibodies in a cocktail of PBS + 0.05% Triton X (PBST) + 10% goat serum. Calculate 30 µL of primary antibody cocktail per cross section.
10. Apply secondary antibody cocktail to the slides for 1 h.
11. Wash slides with PBS + 0.05% Triton X (PBST) for 10 min and with new PBST for another 5 min.
12. Apply a DAPI nuclear staining kit (preferably read-to-use solutions) for 15 min or according to the manufacturer's instructions to stain cell nuclei.
13. Wash slides briefly with PBS + 0.05% Triton X (PBST) for 1 min. Afterward, let slides air-dry briefly.
14. Apply fluorescent mounting medium and coverslips. Store at 4 °C protected from light and perform imaging optimally within 24-48 h.

3. Microscopy

1. Load slides into slide scanner. A maximum of 12 slides is feasible depending on the slide scanner.
2. Start a new project. For preview set the DAPI channel and the 2.5X lens. For the detailed analysis use DAPI, GFP, FITC and Cy5 channels and the 20X objective. Autofocus is acquired throughout the project in the DAPI channel.
3. Use the following colors for each channel: DAPI -blue, FITC: green, Texas Red: red, Cy5: yellow.
4. Create previews of each slide using the DAPI channel. For this purpose, apply manual focus on the first specimen and use it throughout the preview analysis to acquire preview images of every specimen.
5. Outline each cross-section that should be included for the detailed acquisition process. Do not draw outlines close to the edges of the specimen, to ensure caption of the entire area of interest.
6. Set exposure times for each channel. In most cases, exposure parameters of the different channels are identical for all cross-sections.

7. Run automatic image acquisition. Check correct acquisition for the first field of views, to prevent incorrect acquisition early in the automated process.
8. If required, establish remote control using desktop mirroring software. This allows the remote monitoring of the image acquisition process by mirroring the computer screen of the slide scanner to a different computer via any network or internet connection. Although, no changes can be made to the physical setup regarding the used slides, the virtual environment of the image acquisition software allows monitoring the correct focus or exposure times of the images. In addition, the estimated time for completion can be checked regularly.
9. After completion of the image acquisition, control the correct autofocus of all images by manually controlling if the fiber architecture, individual fibers and the various fiber types are distinguishable.
10. Re-acquire incorrectly focused image areas for single fields of view or entire areas of interest. For reacquisition of single areas, mark areas or images throughout the entire project and reacquire areas with manual focus. In most cases, an additional image detail in a different focus level leads to the incorrectly focused images
11. For every cross section that should be included in the final analyses, export each channel (excluding DAPI) separately as jpeg files. Name and sort files according to the exposed channels in folders named Texas Red, FITC and Cy5.

4. Automated Fiber Analysis

NOTE: The macro can be obtained from the following web page: https://www.meduniwien.ac.at/hp/bionicreconstruction/macro/

1. Preview each image using a conventional image editing software before analyses and check for sufficient contrast between stained fibers and background. If necessary, adjust contrast and brightness to increase the difference, using brightness and contrast adjustment commands.
2. Open ImageJ or Fiji. Open the macro using the command "Plugins - Macros - Edit." This shows the macros source code and allows quick adaption of parameters. If necessary, change folder directory for each channel in the source code of the macro.
   NOTE: Values for muscle fiber analyses are based on rat biceps and lumbrical muscles, other species or muscles may require different values.
3. Use the "run" command to start the macro. All images of the folder are now loaded into the macro and quantified in a consecutive order. The results are shown in a new window. This step may take from seconds to several hours, depending on the amount of data being analyzed.
4. Export the results window as a spreadsheet file. Identify values for positively counted Cy5 (slow), FITC (intermediate) and Texas Red (fast) fibers and sum up for total fiber number.

Representative Results

Whole rat muscle cross sections were stained rapidly using immunohistochemistry to identify MHC I, IIA and IIB muscle fibers. Using a fluorescent microscope slide scanner, entire cross sections were then automatically acquired for automated muscle fiber analyses with ImageJ. The concept of the procedure is based on providing a simple, reliable and time-efficient workflow for quantification of muscle fibers.

The procedure's workflow (**Figure 1**) starts with the correct removal and freezing of muscle samples, as previously described in a previous tutorial by Meng *et al.*[27]. This is essential for adequate staining quality. In the next step, the muscle samples are cut and stained using primary antibodies against myosin heavy chain proteins class I, IIA and IIB and appropriate secondary fluorescent antibodies. For optimal staining quality, the manufacturer's recommended concentrations for the secondary antibodies were doubled and Triton X used for the antibody cocktails to provide even staining quality. The immunofluorescent staining of muscle fibers is quick with minor cross-reactivity and can be accomplished in approximately 5 h for batches of up to 26 slides in our setup. Resulting images showed high contrast between positive fibers and surrounding tissue and a strong distinction between different fiber types (**Figure 2**). Next, image acquisition is performed using a slide scanner that permits automatic scanning of entire muscle cross sections. Here, up to 12 slides are scanned automatically, for example, overnight. Hereby, overview images of the cross sections are created in high detail with a resolution that permits inspection of single fiber morphology (**Figure 2**).

In the final step, individual channels of each cross section are exported for quantification of muscle fiber populations using a specifically designed macro for ImageJ. The automatic analysis takes approximately 5-10 min for a rat biceps cross section containing between 3,000-6,000 fibers, whereas the manual analyses took approximately 60 min[25]. Automatic analyses depend strongly on computing power of the workstation and file size. As previously shown, the macro is able to detect relative muscle fiber populations with an accuracy of ± 4% compared to manual analysis (**Figure 3**). Overall absolute counts are higher compared to manual analyses, however, relative counts remained similar within a ±4% range, as previously shown.

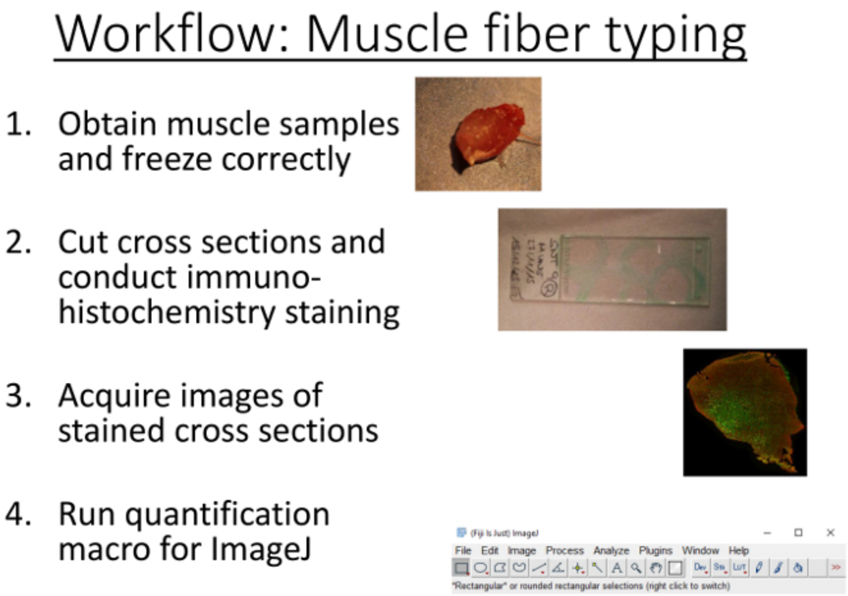

Figure 1: Workflow of the Muscle Fiber Quantification Protocol. First, muscle samples are obtained from the animal and frozen correctly (see JoVE video by Meng *et al.*[27]). Then cut muscle cross sections and conduct immune staining. Acquire images of stained cross sections using a slide scanner for detailed whole cross sections images. Run the quantification macro for ImageJ for muscle fiber population analyses. Please click here to view a larger version of this figure.

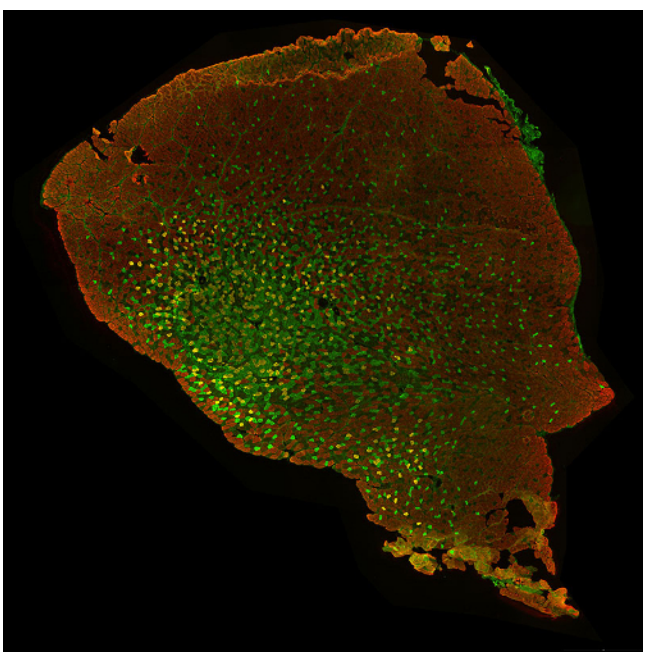

Figure 2: Rat Biceps Muscle Cross Section. Overview of a stained rat biceps muscle cross section: The cross section shows slow MHC I fibers in yellow, intermediate MHC IIA fibers in green and fast MHC IIB fibers in red. Please click here to view a larger version of this figure.

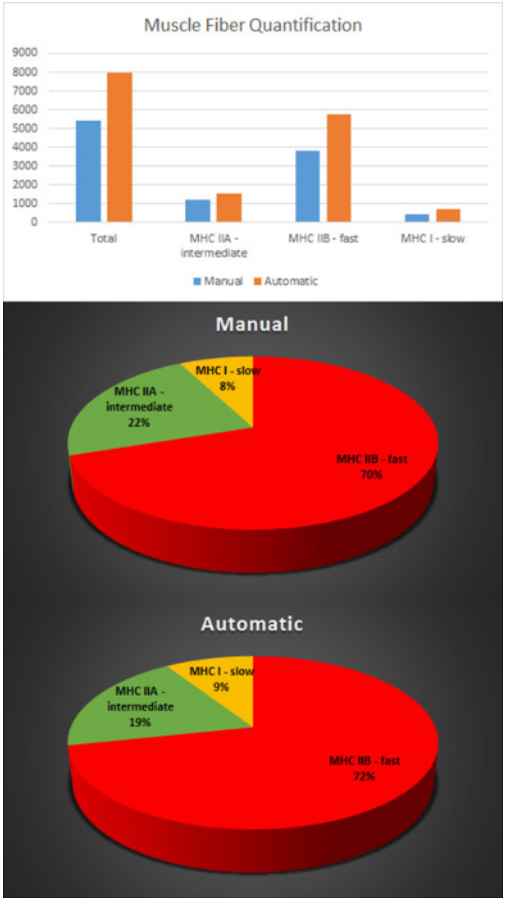

Figure 3: Muscle Fiber Analyses Results of Rat Cross Section in Figure 2. Analyses of muscle MHC I, MHC IIA and MHC IIB fibers. Top: Absolute counts of muscle fibers by manual or automatic count. Middle: Relative portions of muscle fibers populations after manual quantification. Bottom: Relative portions of muscle fibers populations after absolute quantification. Please click here to view a larger version of this figure.

Target structure	Primary AB/ Dilution	Target	Secondary AB/ Dilution	Target	Channel	Color
Cell Nuclei	DAPI		(Staining-Kit)		DAPI	Blue
MHC I (slow)	BA-F8 (1:50)	IgG2b	Fluorophore 633 (1:250)	IgG2b	Cy5	Yellow
MHC IIa (intermediate)	SC-71 (1:600)	IgG1	Fluorophore 488 (1:250)	IgG1	FITC	Green
MHC IIb (fast)	BF-F3 (1:100)	IgM	Fluorophore 555 (1:250)	IgM	Texas Red	Red

Table 1: Immunohistochemical Staining of MHC Fibers. Description of primary and secondary antibodies including dilutions used for the staining procedure. (Modified from Bergmeister *et al.*[25])

Discussion

Here, we demonstrate a widely accessible methodology to study and automatically quantify the muscle fiber populations of rat cross sections via immunohistochemistry in a time efficient manner. For reproducibility, we present a detailed step by step description and potential modifications for applications in other species not described in this study. Furthermore, we discuss the advantages of the procedure, prerequisites for optimal function and its limitations.

Currently, a number of staining methods exist to identify muscle fiber populations and a number of methods have been described for manual or semi-automatic quantification of muscle fibers[17]. However, no protocols exist that combine both and describe a standardized approach to reliably stain muscle fiber populations and automatically acquire and quantify muscle fibers, using freely available analyses software. Based on previous publications[24,29], we optimized existing myosin heavy chain immunohistochemistry staining procedures for automated analyses of fiber populations in murine muscle cross sections. Thereby, we recently showed that the automated analysis of whole cross sections is feasible, reliable compared to manual analyses, and more time-efficient[27]. In addition, the method is user-independent thus reducing human error and thereby providing an objective comparison as for example between different treatment groups.

The whole protocol is based on providing detailed and high-quality images of whole cross sections for the automated quantification process. ImageJ as an open source software provides a number of powerful yet simple commands, which arranged in a macro allow muscle fiber identification. However, quality differences within or between cross section need to be minimized to achieve reliable results. We have identified some critical steps that are essential to achieving the required quality. This includes the use of optimal primary antibodies. Similarly, the use of appropriate secondary antibodies providing distinct fluorescent signals was essential, and we doubled concentrations for improved signal quality. Additionally, we observed a higher and more even staining quality with the use of Triton X in the antibody cocktails. Importantly, this was not observed with the use of Tween instead. Images with suboptimal staining quality were manually adjusted for better contrast to allow automatic image acquisition. Most important for the acquisition process was the correct function of the autofocus system. For this purpose, we used a DAPI-kit to stain the cell nuclei of satellite cells, which provided a reliable landmark for the autofocus system.

For the ImageJ quantification process, optimal values were determined by comparing manual to automated results and consecutive fine-tuning of the parameters[25]: Threshold and Analyze particles. The resulting values were sufficient for biceps and lumbrical muscles and therefore potentially other rat muscles. Additionally, Bloemberg *et al.*[24] have shown that the staining is applicable for other species such as humans or mice. Therefore, the entire protocol can theoretically be adapted for the use in other species, but would, however, require identifying the necessary parameters. The relevant commands are highlighted in the source code.

Further possible modifications of the procedure include the setup of the slide scanner. In our setup, we used a slide scanner system, with a 2.5X lens for the preview and 20X lens for the actual acquisition. Whereas changing the lens for the preview process will provide no significant benefit in terms of quality or speed, the acquisition can also be conducted using a 10X lens to increase the speed (and consequently decrease the resolution) or instead a 40X lens for increasing the resolution and consequently acquisition time. As is shown in **Figure 2**, increasing the resolution is most likely not necessary for rat muscles but may be considered for other species. Other modifications that are suitable in this setup are staining for MHC-IIX fibers or laminin to visualize the muscle's extracellular matrix. These modifications, however, require changing the acquisition setup for two additional reflectors (for example Cy7) and modifying the ImageJ macro to include additional analyses with the correct parameters to the script code.

A limitation of this technique is that the absolute fiber counts were significantly higher compared to manual counts in a previous analysis, although relative counts remained similar[25]. This is most likely the result of inadequate separation and thus repeated quantification of fibers. This effect is more present in fibers that are inadequately stained or show other types of artifacts. However, the number of relative fiber counts and thus fiber populations remained highly accurate. In comparison to the common practice of quantifying a defined sub-portion of a cross section, *e.g.* 40%, we believe that analyses of the entire cross section by the ImageJ are more accurate as a result of the heterogenic muscle fiber architecture (**Figure 2**). A potential limitation of the procedure is the requirement to use expensive slide scanners for automated acquisition of entire cross section, which are not generally available. An alternative approach is to acquire single field of views and quantify each individually by the macro.

In conclusion, we believe this protocol is widely accessible and allows the fast and reliable quantification of whole muscle cross sections using freely available analyses software. Therefore, the introduced procedure provides a useful contribution to the analyses of muscle fiber morphology.

Disclosures

The authors have nothing to disclose.

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References

1. Kung, T. A. *et al.* Motor Unit Changes Seen With Skeletal Muscle Sarcopenia in Oldest Old Rats. *J Gerontol A Biol Sci Med Sci.* **69** (6), 657-665 (2014).
2. Greising, S. M., Medina, J. S., Vasdev, A. K., Sieck, G. C., & Mantilla, C. B. Analysis of muscle fiber clustering in the diaphragm muscle of sarcopenic mice. *Muscle Nerve.* (2015).
3. Claflin, D. R. *et al.* Effects of high- and low-velocity resistance training on the contractile properties of skeletal muscle fibers from young and older humans. *J Appl Physiol.* **111** (4), 1021-1030 (2011).
4. Miller, A. I., Heath, E. M., Dickinson, J. M., & Bressel, E. Relationship Between Muscle Fiber Type and Reactive Balance: A Preliminary Study. *J Mot Behav.* 1-6 (2015).
5. Song, Y., Forsgren, S., Liu, J.-X., Yu, J.-G., & Stål, P. Unilateral Muscle Overuse Causes Bilateral Changes in Muscle Fiber Composition and Vascular Supply. *PLoS ONE.* **9** (12), e116455 (2014).
6. Hopker, J. G. *et al.* The influence of training status, age, and muscle fiber type on cycling efficiency and endurance performance. *J Appl Physiol (1985).* **115** (5), 723-729 (2013).
7. Pette, D., & Staron, R. S. Myosin isoforms, muscle fiber types, and transitions. *Microsc Res Tech.* **50** (6), 500-509 (2000).
8. Suga, T. *et al.* Muscle fiber type-predominant promoter activity in lentiviral-mediated transgenic mouse. *PLoS One.* **6** (3), e16908 (2011).
9. Wang, J. F., Forst, J., Schroder, S., & Schroder, J. M. Correlation of muscle fiber type measurements with clinical and molecular genetic data in Duchenne muscular dystrophy. *Neuromuscul Disord.* **9** (3), 150-158 (1999).

10. Rader, E. P. *et al.* Effect of cleft palate repair on the susceptibility to contraction-induced injury of single permeabilized muscle fibers from congenitally-clefted goat palates. *Cleft Palate Craniofac J.* **45** (2), 113-120 (2008).
11. Macaluso, F., Isaacs, A. W., & Myburgh, K. H. Preferential type II muscle fiber damage from plyometric exercise. *J Athl Train.* **47** (4), 414-420 (2012).
12. Lieber, R. L., & Fridén, J. Clinical significance of skeletal muscle architecture. *Clin. Orthop. Relat. Res.* **383** 140-151 (2001).
13. Schiaffino, S. Fibre types in skeletal muscle: a personal account. *Acta Physiol (Oxf).* **199** (4), 451-463 (2010).
14. Bottinelli, R., Betto, R., Schiaffino, S., & Reggiani, C. Unloaded shortening velocity and myosin heavy chain and alkali light chain isoform composition in rat skeletal muscle fibres. *J Physiol.* **478** (Pt 2), 341-349 (1994).
15. Schiaffino, S., & Reggiani, C. Myosin isoforms in mammalian skeletal muscle. *J Appl Physiol (1985).* **77** (2), 493-501 (1994).
16. Larsson, L., & Moss, R. L. Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. *J Physiol.* **472** 595-614 (1993).
17. Kostrominova, T. Y., Reiner, D. S., Haas, R. H., Ingermanson, R., & McDonough, P. M. Automated methods for the analysis of skeletal muscle fiber size and metabolic type. *Int Rev Cell Mol Biol.* **306** 275-332 (2013).
18. Schiaffino, S. *et al.* Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Motil.* **10** (3), 197-205 (1989).
19. Lieber, R. L. *Skeletal muscle structure, function, and plasticity.* Lippincott Williams & Wilkins Baltimore, MD (2009).
20. Hintz, C. S., Coyle, E. F., Kaiser, K. K., Chi, M. M., & Lowry, O. H. Comparison of muscle fiber typing by quantitative enzyme assays and by myosin ATPase staining. *J Histochem Cytochem.* **32** (6), 655-660 (1984).
21. Havenith, M. G., Visser, R., Schrijvers-van Schendel, J. M., & Bosman, F. T. Muscle fiber typing in routinely processed skeletal muscle with monoclonal antibodies. *Histochemistry.* **93** (5), 497-499 (1990).
22. Likar, B., & Pernuš, F. Registration of serial transverse sections of muscle fibers. *Cytometry.* **37** (2), 93-106 (1999).
23. Liu, F. *et al.* Automated fiber-type-specific cross-sectional area assessment and myonuclei counting in skeletal muscle. *J Appl Physiol (1985).* **115** (11), 1714-1724 (2013).
24. Bloemberg, D., & Quadrilatero, J. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One.* **7** (4), e35273 (2012).
25. Bergmeister, K. D. *et al.* Automated muscle fiber type population analysis with ImageJ of whole rat muscles using rapid myosin heavy chain immunohistochemistry. *Muscle Nerve.* (2016).
26. Guillen, J. FELASA guidelines and recommendations. *J Am Assoc Lab Anim Sci.* **51** (3), 311-321 (2012).
27. Meng, H. *et al.* Tissue Triage and Freezing for Models of Skeletal Muscle Disease. *J Vis Exp* (89), e51586 (2014).
28. Guillen, J. FELASA Guidelines and Recommendations. *J Am Assoc Lab Animal Sci*. **51** (3), 311-321 (2012).
29. Ribarič, S., & ČebaŠek, V. Simultaneous Visualization of Myosin Heavy Chain Isoforms in Single Muscle Sections. *Cells Tissues Organs.* **197** (4), 312-321 (2013).